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DIAGNOSIS OF (AIDS) RELATED INTESTINAL PARASITES

ANNUAL/FINAL REPORT

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JUNE 20, 1990

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Supported by

U.S. ARMY MEDICAL RESEARCH AND DEVELOPMENT COMMAND
Fort Detrick, Frederick, Maryland 21701-5012

MIPR 86MM6514

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SECURITY CLASSIFICATION OF THIS PAGE

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1a. REPORT SECURITY CLASSIFICATION Unclassified	1b. RESTRICTIVE MARKINGS
2a. SECURITY CLASSIFICATION AUTHORITY	3. DISTRIBUTION/AVAILABILITY OF REPORT Approved for public release; distribution unlimited
2b. DECLASSIFICATION/DOWNGRADING SCHEDULE	
4. PERFORMING ORGANIZATION REPORT NUMBER(S)	5. MONITORING ORGANIZATION REPORT NUMBER(S)
6a. NAME OF PERFORMING ORGANIZATION Uniformed Services University of the Health Sciences 6b. OFFICE SYMBOL (If applicable)	7a. NAME OF MONITORING ORGANIZATION
6c. ADDRESS (City, State, and ZIP Code) F. Edward Herbert School of Medicine 4301 Jones Bridge Road Bethesda, Maryland 20814-4799	7b. ADDRESS (City, State, and ZIP Code)
8a. NAME OF FUNDING/SPONSORING ORGANIZATION U.S. Army Medical Research & Development Command 8b. OFFICE SYMBOL (If applicable)	9. PROCUREMENT INSTRUMENT IDENTIFICATION NUMBER MIPR 86MM6514
8c. ADDRESS (City, State, and ZIP Code) Fort Detrick Frederick, Maryland 21701-5012	10. SOURCE OF FUNDING NUMBERS PROGRAM ELEMENT NO. 63105A; PROJECT NO. 3M2-63105DH29; TASK NO. AB; WORK UNIT ACCESSION NO. 063

11. TITLE (Include Security Classification)

(U) Diagnosis of (AIDS) Related Intestinal Parasites

12. PERSONAL AUTHOR(S)
Beth L.P. Ungar

13a. TYPE OF REPORT	13b. TIME COVERED	14. DATE OF REPORT (Year Month, Day)	15. PAGE COUNT
Annual/Final	FROM 9/26/86 TO 12/31/89	1990 June 20	25

16. SUPPLEMENTARY NOTATION
Annual cover period of time 26 September 1988 - 31 December 1989

17. COSATI CODES			18. SUBJECT TERMS (Continue on reverse if necessary and identify by block number)
FIELD	GROUP	SUB-GROUP	RA 1; Retrovirus; Diarrhea; Parasitology
06	03		
06	13		

19. ABSTRACT (Continue on reverse if necessary and identify by block number)

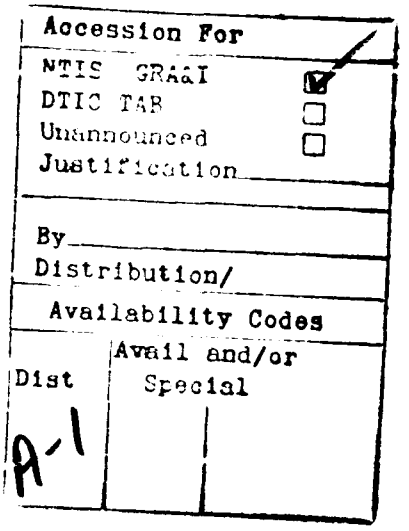
20. DISTRIBUTION/AVAILABILITY OF ABSTRACT	21. ABSTRACT SECURITY CLASSIFICATION
☐ UNCLASSIFIED/UNLIMITED ☒ SAME AS RPT ☐ DTIC USERS	Unclassified
22a. NAME OF RESPONSIBLE INDIVIDUAL Frances Bostian	22b. TELEPHONE (Include Area Code) 301-663-7325
	22c. OFFICE SYMBOL SGRD-RMI-S

DD Form 1473, JUN 86 *Previous editions are obsolete* SECURITY CLASSIFICATION OF THIS PAGE

FOREWORD

In conducting research using animals, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources Commission on Life Sciences, National Research Council (DHHS, PHS, NIH Publication No. 86-23, Revised 1985).

For the protection of human subjects the investigator(s) have adhered to policies of applicable Federal Law 45CFR46.



I. BACKGROUND

During the past decade, Cryptosporidium has become acknowledged as an important human pathogen. Although the first two human cases were only described in 1976 with a half dozen more reported during the next six years, the onset of the aquired immunodeficiency syndrome (AIDS) pandemic led to recognition of Cryptosporidium as an agent of fulminant and potentially life-threatening diarrhea (1-6). Now, there are over 300 scientific publications on human cryptosporidiosis many of which focus on areas of current research: refining diagnostic techniques, understanding pathogenesis and evaluating promising therapies.

From a clinical perspective, diarrhea remains the most noteworthy symptom and it can range from cholera-like and relentless to scant and intermittent. Although in immunologically healthy patients diarrhea characteristically lasts no longer than 30 days before spontaneous cure, in immunodeficient patients (especially those who are HIV-infected) recovery is unlikely unless the cause of immunosuppression is removed. Death associated with malabsorption, dehydration and electrolyte imbalance is not unusual (1-4). In any individual, however, infection can be severe and debilitating with potential for transmission persistent as long as oocysts are shed, up to 60 days even after symptoms abate (7,8).

Recently, other clinical profiles have also received attention. Because diagnostic proficiency varies with laboratory experience and frequency of cryptosporidiosis in a given population, and because Cryptosporidium oocysts are more easily identified in persons with diarrhea than those without, these other clinical presentations may actually be underrecognized: they include acalculous cholecystitis, sclerosing cholangitis, hepatitis, pancreatitis, reactive arthritis and a variety of respiratory symptoms (9-21). While initial reports of these manifestations were mostly in HIV-infected patients, they are increasingly described in immunologically intact persons as well.

Asymptomatic infections are probably underestimated and are likely important in transmission (1,2). For example, in one series in New York, of 154 adult patients without diarrhea undergoing endoscopy, 18 (11.7%) had Cryptosporidium detected in duodenal aspirates; 7 had Cryptosporidium oocysts subsequently identified in stool specimens (22). In another a survey of 22 children attending an upper middle class daycare center in New York where no diarrheal outbreak has been noted, 6 (27%) asymptomatic children excreted oocysts (23). Of 30 randomly chosen children from 30 separate home daycare centers in Grand Junction, Colorado, 9 (30%) excreted Cryptosporidium oocysts, and 6 of these were asymptomatic (24). Whether these represent true asymptomatic infections or oocysts identified sometime after an undiagnosed perhaps brief, mild or atypical clinical illness, remains speculative.

Certain groups are now considered to be at high risk for cryptosporidiosis based on patterns of transmission and patients' immunologic status. Cryptosporidium oocysts need to be orally ingested to cause infection. Person-to-person transmission has been established between household or family members and sexual partners of infected persons, children and caretakers in day-care centers and health care workers and their patients (1,4). Animal-to-person transmission has been described from household pets, laboratory

animals and farm animals (1-4). Immunocompromised patients, particularly HIV-infected persons, but also those who are on exogenous immunosuppressive agents, hypo- or a-gammaglobulinemic patients, malnourished persons, and patients with intercurrent viral infections such as measles or perhaps chickenpox, are especially susceptible (1). Outbreaks -- waterborne, in hospitals, and in daycare centers -- have also been well-documented (1,4). Of increasing importance are nosocomially infected hospitalized patients, exposed family members and health care workers, and travellers or temporary workers in areas of high endemicity.

The function of the human immune response to Cryptosporidium which governs the host's ability to eradicate Cryptosporidium infection is poorly understood. Both cellular and humoral immunity are involved, as evidenced by development of persistent cryptosporidiosis both in HIV-infected patients with T-cell abnormalities and in patients with gammaglobulin deficiencies; this contrasts with development of transient Cryptosporidium infection in immunologically normal individuals. The role of different antibody classes as well as T-helper cells (CD4+), T suppressor cells (CD8+), and T cell products, such as gamma interferon (a regulator of infection with other intracellular pathogens) or interleukin-2 (reported to have cured Cryptosporidium in two patients receiving it as part of an AIDS treatment protocol [25]), remains to be investigated. The importance of immunologic mediators in subsequent protection, either from infection or from symptomatic expression of disease, is also unknown.

Ability to diagnose Cryptosporidium infections rapidly and accurately remains subject to the proficiency of the individual laboratory. Diagnosis of cryptosporidiosis by identification of Cryptosporidium oocysts in human fecal specimens was first accomplished in 1980 using Giemsa staining (26), and subsequently more than a half dozen stool concentration methods and more than a dozen staining techniques have been developed without consensus as to diagnostic dependability (2,4). In the United States, as many as three different techniques may be used in a single laboratory; concentration by flotation in Sheather's sugar solution, modified acid-fast and fluorescent stains, and monoclonal antibody-based fluorescent detection assays are most often used (27-29). All microscopic diagnoses rely on direct visualization and morphologic recognition of small, 4 to 6 micron, oocysts which may be scant in number, intermittently shed, or inconsistently stained (1,2); examination of three or more fecal specimens to detect Cryptosporidium oocysts may be necessary (2). Recent identification of an acid-fast, 8 to 10 micron, blue-green algae as a putative agent of diarrhea may limit utility of acid-fast staining in the future (30).

Treatment of cryptosporidiosis, critical for immunodeficient patients, remains enigmatic. Spiramycin, somatostatin, interleukin-2, azidothymidine, and diclazuril sodium may modify symptoms or produce a subjective feeling of well-being in some HIV-infected or other immunocompromised patients, although none of these agents has predictable efficacy (1,25, 31-35). Only rare anecdotal success has been reported with more than ninety other chemotherapeutic, biologic and other antidiarrheal agents and the mainstay of therapy remains hydration and hyperalimentation (1-4). Bovine-derived products, colostrum hyperimmune to Cryptosporidium, transfer factor from lymph node homogenates of cattle hyperimmune to Cryptosporidium and, perhaps, immunoglobulins purified from hyperimmune colostrum remain the most promising interventional agents (36-40).

From an epidemiologic perspective, Cryptosporidium has been identified as a significant human enteropathogen on all six inhabited continents. There have been more than 100 geographically-based surveys for Cryptosporidium oocyst excretion in at least 40 different countries (1). Although variations in population examined (numbers of specimens versus numbers of patients) render these studies not comparable in the strictest sense, the range and mean prevalence rates of active Cryptosporidium infection (oocyst excretion) in various land areas can nevertheless be summarized: In the more industrialized countries of Europe and North America, prevalence rate, excluding documented outbreaks, is generally between 1 and 3%. In contrast, mean prevalence rates from elsewhere in the world are between 4.9% in Asia and 10.4% in Africa (1). The higher prevalence of cryptosporidiosis in some less developed countries may relate to relative lack of clean water and sanitary facilities, crowding of households, and large number of animals in close proximity to residences. In general, urban areas in lesser developed countries have more cryptosporidiosis than less crowded rural areas, while the reverse appears to be true in more developed countries.

Both waterborne and nosocomial outbreaks are becomming epidemiologically more significant. Three probable nosocomial outbreaks of cryptosporidiosis have been recorded from Italy, South Africa, and Argentina. In the first, six patients on a bone marrow transplant unit in Italy, were infected with Cryptosporidium. One of these six had shared a room with a patient with cryptosporidiosis before being transferred to the transplant unit. No staff member or relative (0 of 22) was found to be infected; water samples from floor cleaning machines and toilet-cleaning rags were heavily contaminated with Cryptosporidium (41). In Durban, South Africa, during a randomized trial of spiramycin in hospitalized children, Cryptosporidium oocysts were found in stools of nine patients not participating in the study up to 2 weeks following an initial negative examination; this was considered consistent with hospital spread (32). In a renal transplant unit in Buenos Aires, Argentina, 11 of 14 patients with acute diarrhea had cryptosporidiosis; hospital transmission was thought possible through shared toilet facilities (42).

The environment, especially water, has been increasingly recognized as an important source for highly resilient Cryptosporidium oocysts (they remain viable at 4°C in the laboratory for up to 12 months, and must be kept at 45°C for 5 to 20 min before they lose infectivity [4,43,44]). In fact, Cryptosporidium has been isolated from drinking water (raw and city finished), river, stream and reservoir water in North America, South America, and Europe and is likely ubiquitous in water throughout the world (45-53); this makes travel, particularly to areas with poorer water supplies, a risk factor for cryptosporidiosis.

The first association of cryptosporidiosis with travel was actually suggested in 1983, when 12 of 14 Finnish patients found to be excreting Cryptosporidium oocysts were retrospectively discovered to have become ill within 12 days of visiting Leningrad, U.S.S.R., and to have had no contact with each other (54). In a subsequent prospective study, three groups of Finnish students were screened for Cryptosporidium before and after a visit to Leningrad and 9 of 34 students became infected (55). Additional travel-related cases have been reported in visitors to Egypt, the Central African Republic, Mexico, St. Lucia, Pakistan and New Guinea (56-59).

Several common-source waterborne outbreaks have been reported. An outbreak of gastroenteritis with a 34% attack rate in a residential neighborhood in San Antonio, Texas, in 1984, has been linked to fecal contamination of the community water supply; 60% (47/79) of the residents of this area compared to 6% (12/194) of non-residents excreted Cryptosporidium (46). Exposure to surface water by drinking or swimming was implicated in 78 cases of cryptosporidiosis in New Mexico in 1986 (45,47). In Sheffield, Great Britain, in 1986, 81% (84/104) of patients with cryptosporidiosis drank water from a single reservoir complex; oocysts were found in fecal samples in cattle grazing near the reservoir, in reservoir surface water and feeding streams, and in five healthy trout swimming in the reservoir (49). In Scotland, in 1988, 27 persons with Cryptosporidium infection all drank from the same water supply; organisms were found in treated water in samples from the treatment plant, and from stream water feeding into the treatment plant (51).

In the largest and best-documented waterborne outbreak reported to date, approximately 13,000 individuals developed diarrhea in Carrollton County, Georgia, during a 4-week period in the winter of 1987 (48). In a retrospective random telephone survey, 61% (299/489) of household members drinking from the public water supply, compared to 20% (64/322) of unexposed persons, were symptomatic; 17% (56/266) nursing home residents on the municipal water supply had diarrhea compared to 0/27 drinking well water. Fifty-eight of 147 fecal specimens from patients with gastroenteritis contained Cryptosporidium oocysts; one commercial laboratory serving the area had 0/236 fecal specimens positive for Cryptosporidium in December and 20/293 specimens positive in January, all from the affected area. The prevalence of anti-Cryptosporidium IgG two weeks or more after symptoms was significantly higher in symptomatic telephone respondents than among non-resident controls. Although the water treatment plant was appropriately meeting all regulatory agency purification standards, Cryptosporidium oocysts were found in treated public water and the outbreak was terminated by modification in the water-treatment process (backwashing of filters, using variable-speed flocculators, changing means of assessing turbidity). Cryptosporidium excreted from cattle in the watershed area and/or sewage overflow were probably responsible for water contamination. It is likely that as diagnostic techniques continue to improve, more waterborne outbreaks of diarrhea will be associated with Cryptosporidium.

Epidemiologic data on the relationship between cryptosporidiosis and HIV-infected patients show that in 1986, in the United States, an estimated 3.6% of late-stage HIV-infected patients had cryptosporidiosis at time of report to the Centers for Disease Control (60). However, in patients with AIDS and diarrhea, 15% of those evaluated at the National Institutes of Health, Bethesda, MD, and 16% of those evaluated at the Johns Hopkins Hospital, Baltimore, had Cryptosporidium infection, the most common pathogen in the latter series (61,62). In a retrospective review of 100 charts from patients treated at Montefiore Medical Center or North Central Bronx Hospital, New York, Cryptosporidium was identified in 5 of 36 (14%) AIDS patients with diarrhea (63). In two hospitals in Great Britain and Brazil, 11 and 12% of AIDS patients, respectively, had cryptosporidiosis, and 19% of those in Great Britain were thought to have died as a direct result (33,64). In a series from Paris, 21.1% of 132 prospectively studied AIDS patients were infected with Cryptosporidium, the most frequent enteropathogen identified (65). Finally, 48.7% of 23 AIDS patients with enteropathy in one hospital study from Uganda had cryptosporidiosis. As the number of late-stage HIV-infected persons increases,

there will likely be more cryptosporidiosis in the overall HIV-infected population. It will become increasingly imperative to have unambiguous diagnostic capabilities as well as successful prophylactic and therapeutic interventional agents for Cryptosporidium infection.

II. FINAL RESULTS

A. Development of an ELISA to Detect Cryptosporidium Antigens in Fecal Specimens.

A double antibody indirect ELISA test was developed to detect Cryptosporidium antigens in fecal specimens (66). Reagents for this assay were produced by: (1) oral infection of colostrum - deprived calves with Cryptosporidium and collection of fecal output (USDA collaboration); (2) purification of Cryptosporidium oocysts from calf feces for use as immunogens by flotation in distilled water saturated with sodium chloride, with subsequent sedimentation in distilled water, washing in sodium hypochlorite, and sonication; (3) immunization of rabbits and goats, screened negative for specific anti-Cryptosporidium antibodies, with purified Cryptosporidium over an eight-month period to produce high-titered specific antisera. Optimal antibody combinations as well as concentrations for the ELISA test were determined by checkerboard titration using either purified Cryptosporidium oocysts or Cryptosporidium oocysts in calf feces as antigen.

The following ELISA procedure was established using standard incubation times and washing procedures. ELISA microtiter plates were prepared by coating alternate double rows of wells with 100 µl of 1 1:10,000 dilution of either immune or non-immune rabbit antisera in carbonate buffer (pH 9.6). Plates were stored at 4°C for at least 14 hours before use. Test material was prepared by mixing 25 µl of antigen (feces homogenized in phosphate buffered saline [PBS] with 75 µl of PBS-Tween 20 - 0.5% gelatin (PBS-T-G). This was added to 4 wells, two coated with immune and two with non-immune rabbit antisera, which ultimately minimizes non-specific color reactivity. Immune goat antisera diluted 1:400 in PBS-T-G was the second antibody used, and commercial enzyme-conjugated anti-goat immunoglobulin and substrate completed the assay.

When fecal specimens were tested, at least five negative and one positive control samples were applied to each plate to allow standardization between microtiter plates. To calculate results, as done previously in ELISA tests to detect antigens of either Giardia lamblia or Entamoeba histolytica in human fecal specimens (67,68), the mean optical density (O.D.) reading of wells coated with non-immune rabbit sera was subtracted from the mean of those coated with immune sera to give a specific O.D. for a clinical specimen. The mean and standard deviation for the negative control specimens were calculated as above and a clinical specimen was considered positive if its specific O.D. value was 0.08 units greater than the mean O.D. plus 2 standard deviations of the negative controls. When different dilutions of purified Cryptosporidium oocysts were tested, a dilution was considered positive if it yielded a value greater than the buffer alone.

244 fecal specimens were collected: 231 were from 113 persons, aged 6 months to 60 years, presumed or known to be seronegative for human immunodeficiency virus (HIV) in the United States and in an area endemic for cryptosporidiosis in Peru. An additional 13 fecal samples were from HIV seropositive patients with diarrhea. Specimens for ELISA testing were refrigerated or placed on ice immediately after collection and frozen at -70°C within 8 hours. They were thawed immediately prior to use and heated to 100°C in a water bath for two minutes to inactivate bacterial proteolytic enzymes. Original fecal specimens were immediately refrozen at -70°C. Each specimen was examined between two and four times on different microtiter plates and in duplicate on each microtiter plate.

Fresh fecal specimens were examined in the laboratory of original submission by direct microscopy after concentration and/or after modified acid-fast or fluorescent monoclonal antibody staining. Cryptosporidium was considered a definite diagnosis if organisms were found in two different laboratories or using two different staining processes (modified acid fast or fluorescent monoclonal antibody). 62 specimens (12 from HIV seropositive patients) had cryptosporidiosis; for the remaining 182 specimens, confirmed identification (two techniques or two laboratories) of Cryptosporidium could not be made. Fecal specimens were also examined in the laboratory of original submission for other parasites using standard techniques. Eight other intestinal parasites were identified Giardia lamblia (67 specimens), Endolimax nana (38 specimens), Entamoeba coli (34 specimens), Chilomastix mesnili (27 specimens), Ascaris lumbricoides (8 specimens), Hymenolepis nana (7 specimens), Trichuris trichiura (4 specimens) and Isospora belli (3 specimens).

To determine the ability of ELISA to detect known numbers of Cryptosporidium oocysts, serial two-fold dilutions containing from 2 to 500,000 purified unsonicated Cryptosporidium organisms per 100 µl PBS buffer were examined in duplicate. The ELISA was easily able to detect between 2000 and 4000 purified Cryptosporidium oocysts on 6 comparable ELISA tests run on different days. It was able to detect between 1000 and 2000 purified Cryptosporidium oocysts on two additional ELISA tests performed on other days.

For human fecal specimens, 51 of 62 specimens from patients with confirmed cryptosporidiosis were positive by ELISA. All of the 11 remaining specimens of this group came from patients with fewer than 5 oocysts per 100 µl of concentrated fecal specimen and 10 of these were from Peruvians. The inability to detect Cryptosporidium in these specimens may suggest that they contained antigen that was inaccessible to or not recognized by the detecting polyclonal antibodies. The polyclonal antibodies were raised to sonicated Cryptosporidium oocysts and therefore, to both sporozoites and oocyst wall components. In an infected individual, these or other antigens may be present and detectable at different life cycle stages of the parasite. Another possibility, particularly in the Peruvian patients, is that some Cryptosporidium isolates may be antigenically distinct from the isolates from the United States and used to produce antisera, or that Peruvian patients may not have had Cryptosporidium parvum infection at all but rather ingested, perhaps through a contaminated water supply, another possibly antigenically distinct Cryptosporidium isolate such as C. baileyi. Some specimens from Peru may have thawed during transport and lost recognizable antigen. Alternately, the inability of the ELISA to detect Cryptosporidium antigens from these 11 patients or generally from fewer than 1000 to 2000 purified oocysts may simply mean that these samples contained an amount of free antigen below the sensitivity of the assay.

Of 182 samples from patients without confirmed cryptosporidiosis, 176 were negative by ELISA. Three of the six ELISA-positive microscopy-negative specimens came from persons with cryptosporidiosis 30 to 60 days earlier, suggesting either that microscopically undiagnosed Cryptosporidium was still present or that the ELISA detected disintegrating organisms or their products. Two other specimens of this group came from persons with only one unconfirmed positive microscopic examination which may imply real infection or an ability to detect free antigen from some extracellular life cycle stage. In these cases, the ELISA may have detected cryptosporidiosis more efficiently then other means

of diagnosis, and may be important in identifying persons not actively excreting oocysts at time of specimen collection which is relevant to transmission of infection. There was no medical history or additional specimen available from the final patient in this group.

Compared to microscopic examination, the overall sensitivity of the assay was 82.3%. Specificity was 96.7%. The predictive value of a positive ELISA was 89.5% and the predictive value of a negative ELISA was 94.2%. Limits on the ability of this ELISA to detect Cryptosporidium antigen include: not all ELISA-positive specimens retained detectable Cryptosporidium antigen after three freeze/thaw cycles over a 6 month period: for four positive specimens, positive ELISA readings turned negative by 37 days (earliest) to 158 days (latest) after initial testing. For an additional six specimens, however, positive ELISA readings remained positive for at least 21 days (last time tested) to 164 days (last time tested) for different specimens. Results for 16 ELISA-negative specimens were not affected by at least three freeze/thaw cycles and ELISA tests for as long as 56 days. Presence of additional parasites did not affect positivity of ELISA results for specimens with microscopically-diagnosed Cryptosporidium or negativity for specimens without Cryptosporidium. Preservation in 10% formalin or in 2.5% potassium dichromate did destroy ability of ELISA to detect Cryptosporidium antigens.

This ELISA for Cryptosporidium antigen detection in fecal specimens offers a diagnostic alternative to direct microscopy and represents the first generation of an ELISA test for this organism. In terms of personnel, time and economy compared to using at least two microscopic techniques to diagnose infection, this ELISA test is quite practical. Samples are easy to prepare for testing and, although a limiting feature may be the inability to use this ELISA when specimens are preserved in formalin, this problem has been circumvented in later generations of ELISA tests for Giardia lamblia (69,70). In terms of accuracy, the assay may eliminate some of the skill needed in performing complicated staining procedures and in recognizing the morphology of the small Cryptosporidium oocyst, particularly important because of the recent identification of an acid-fast algae-like intestinal organism associated with prolonged diarrhea (30). Future generations of an ELISA test to detect Cryptosporidium antigens may be improved by using polyclonal or monoclonal antibodies raised to ubiquitous immunogenic Cryptosporidium antigens, or by using a pool of monoclonal antibodies with different specifications.

Parenthetically, our laboratory was also involved this year in showing that another small acid-fast organism associated with diarrhea was not Cryptosporidium (30). The organism is 8.0 to 10.0 microns in diameter (2.0 to 4.0 microns larger than Cryptosporidium), and, like Cryptosporidium, floats in Sheather's solution, and stains red with the modified acid-fast stain. It was found in the stools of 55 immunocompetent patients who presented to the CIWEC Traveller's Clinic in Kathmandu, Nepal, from June to November 1989, and was associated with an illness characterized by prolonged watery diarrhea (43± 24 days mean duration; range 4 to 107 days), anorexia, fatigue and weight loss. The organism was examined in our laboratory by microscopy after Sheather's flotation, modified acid-fast staining and fluorescent monoclonal antibody tagging and was found to be morphologically dissimilar to Cryptosporidium without reaction to specific commercially available monoclonal antibodies (Merifluor. Meridian Diagnostics, Cincinnati, Ohio). Identification of the organism as a blue-green algae was made at the Centers for Disease Control by

electron microscopy. Since clinical illness may mimic cryptosporidiosis and since the commonly used modified acid-fast stain may lead to misidentification of this algae as Cryptosporidium if exact measurements are not taken, preferred diagnostic techniques for cryptosporidiosis are likely to need re-evaluation in the near future.

B. Seroepidemiologic Studies of Cryptosporidium Infections in Humans

Using a previously developed ELISA to detect anti-Cryptosporidium IgM or IgG (71), work was completed assessing seroprevalence of Cryptosporidium in Peru and Venezuela (52). The ELISA was used to examine randomly selected sera from 389 children and adults in Lima, Peru, and 84 children in Maracaibo and Caracas, Venezuela. In Peru, 19.8%, and in Venezuela, 15.5% of the study population was positive for specific IgG and IgM simultaneously, consistent with active or recent infection, and representing a larger percent than normally reported from stool examinations of individuals seeking medical attention. Sixty-four percent from each country had detectable anti-Cryptosporidium IgG, suggesting in economically disadvantaged regions such as these, the majority of residents have been infected sometime in life. Detection of specific IgG increased in the 2-3 year-old group, indicating this is a common age for infection. Persistence of IgG and less often, IgM, antibody response occurred over 12 months in some individuals, although whether or not this protects from or modifies the nature of the infection or symptoms needs further investigation. These findings suggest that Cryptosporidium infections are endemic in the communities surveyed and that most residents have been infected.

Using the same ELISA test, sera from 75 U.S. Peace Corps volunteers were examined in an effort to define the prevalence of Cryptosporidium infection in healtny U.S adults, and to determine how often Cryptosporidium infection occurs after relocation to a situation of potentially great exposure (72). 32% of volunteers had detectable anti-Cryptosporidium IgG initially, suggesting that in the United States as well, infection frequently occurs. After 6 weeks, 1 year or two years overseas, 5%, 14% and 13.6% of seronegative individuals respectively became newly IgG positive. Of 22 volunteers followed for two years, only 2 showed no specific immune response to Cryptosporidium during this time. This implies that the risk of infection is real for travellers and temporary workers in highly endemic areas.

Finally, at the request of the Centers for Disease Control in conjunction with their retrospcetive evaluation of a presumed waterborne outbreak of Cryptoporidium-associated diarrhea in Carrollton County, Georgia (48), serum specimens were evaluated from (1) 20 presumed healthy CDC employees; (2) 39 Carrollton County residents with gastrointestinal symptoms and Cryptosporidium detected on stool examination; (3) 37 symptomatic Carrollton County residents with no Cryptosporidium organisms detected on stool examination; and (4) 30 asymptomatic and 78 symptomatic Carrollton County residents without any fecal examinations. IgM and IgG antibody to Cryptosporidium were simultaneously detected more often in symptomatic Carrollton County residents (14.6%) than in asymptomatic residents (5%) or presumed healthy CDC employees (5%), consistent with recent infection in the symptomatic individuals. For residents actively excreting oocysts, 76.9% had specific IgG 14 or more days after onset of symptoms, but only 27.2% earlier than 14 days; for residents not excreting oocysts, 55% had specific IgG irrespective of time from onset of symptoms. IgG antibody to Cryptosporidium alone was detected in 35% of the healthy CDC

employees, a percentage comparable to the healthy Peace Corps volunteers. These serologic studies helped confirm the likelihood that Cryptosporidium was the agent of diarrhea in this outbreak. They further suggest Cryptosporidium infections may be more common than realized in the U.S. with potential pockets of endemnicity. (In Carrollton County, the surface water supply to the water treatment plant was likely contaminated by infected cattle; the water treatment plant was basically meeting all water quality standards which were inadequate to remove the Cryptosporidium, circumstances probably duplicated elsewhere).

C. Evaluation of Therapeutic Modalities for Cryptosporidium Infection

The possibility that hyperimmune bovine colostrum may be therapeutically useful in humans at least in temporary clinical improvement if not in parasitologic cure has been suggested (38). We have participated in several studies evaluating the ability of colostrum to protect against Cryptosporidium infection. In one set of experiments using a mouse model, mice suckling from dams hyperimmunized both orally and parenterally with Cryptosporidium parvum oocysts were not protected from infection (73). Immune dams produced serum antibody against C. parvum while non-immune control dams did not; anti-Cryptosporidium IgA and IgG were demonstrated in milk whey extracted from the stomachs of mice suckling immune dams but not in milk whey from mice suckling non-immune dams. In a second set of experiments, bovine colostrum specifically hyperimmune to Cryptosporidium was produced by parenteral injection and intramammary infusion of oocysts into pregnant dairy cows (USDA collaboration). The hyperimmune state was indicated by titers > 1:200,000 of Cryptosporidium bovine immunoglobulins (IgG-1, IgM and IgA) detected by ELISA in our laboratory. This colostrum has been used to assess protective efficacy in neonatal calves: calves fed hyperimmune colostrum prior to oral infection with Cryptosporidium had significantly less diarrhea ($p < 0.02$) and shed oocysts for significantly less time ($p < 0.05$) than the control group (74). It has also been used compassionately to treat two HIV-infected persons in whom it lead to a two-to-three month total remission of symptoms (40).

Using an adult athymic nude (nu/nu) mouse model of chronic symptomatic cryptosporidiosis (75), efficacy of hyperimmune bovine colostrum as well as of the chemotherapeutic agent diclazuril sodium (currently being evaluated in clinical trial in New York) were assessed as interventional agents: Approximately 10^7 Cryptosporidium parvum oocysts were fed to adult athymic nude outbred mice (Small Animal Division, National Cancer Institute, National Institutes of Health) on two days (one day rest) by gastric gavage. All mice received a subcutaneous dose of 12 mg sulfadimethoxin on days of gavage. Each animal was weighed prior to infection and weekly therafter. Any clinical abnormalities were described. Fecal pellets were examined from each mouse following concentration for Cryptosporidium oocysts both prior to inoculation and twice a week thereafter. Stool consistency was noted at each collection.

Efficacy of hyperimmune bovine colostrum was assessed in three temporally unrelated experiments (all animal groups contained approximately 5 mice). In the first experiment, animals were left untreated or were fed approximately 500 cc of bovine colostrum hyperimmune to Cryptosporidium by oral gavage on 5 of 7 days beginning on the first day after infection and lasting until day 108 of infection. While there was no difference in pooled mean number of oocysts shed between the treated and untreated group before colostrum administration, during

treatment 2.1 oocysts were detectable per 5 high power (45x) microscopic fields in the untreated group compared to 0.8 in the treated group. Animals were followed for an additional ninety days after treatment without significant clinical difference between groups.

A second comparable experiment showed that during the treatment period from day two to day 127 after infection, the pooled mean number of oocysts shed in the treated group was 3.4 oocysts per 5 high power (45x) microscopic fields versus 6.4 oocysts in the untreated group; after treatment during more than 100 days of follow-up, the comparable numbers were 0.57 oocysts in the treated group compared to 15.1 oocysts in the untreated group. One treated mouse in whom oocyst shedding disappeared completely was followed for more than one year at which time necropsy did not show any residual infection. A final experiment compared efficacy of two batches of hyperimmune colostrum prepared at different times. During the treatment period from day 1 to day 100 after infection, the group receiving the colostrum used both in the previous two experiments and in compassionate treatment of two HIV-infected patients shed a mean of 3.2 oocysts per 5 high power (45x) microscopic fields compared to 16.7 oocysts for the group receiving the second batch. This suggests that all bovine hyperimmune colostrum to Cryptosporidium is not necessarily equally efficacious despite comparable levels of ELISA-detectable specific anti-Cryptosporidium antibody. The biologically active factor(s) need further characterization.

For evaluation of diclazuril sodium, twenty-five days afer initial inoculation with Cryptosporidium, when all animals were persistently shedding oocysts, treatment with free-base diclazuril sodium (provided by Barbara Laughon, Ph.D., Division of AIDS, NIAID, NIH) was begun. Mice were randomized into three groups of 6 or 7 animals each. Group 1 (n=6) received 4 mg diclazuril mixed in 250 µl of distilled water; group 2 (n=6) received 8 mg of drug mixed in 250 µl of distilled water, and group 3 (n=7) received distilled water only. Doses were calculated by Dr. Laughon to be equivalent to a 400 mg and 800 mg human dose, those being evaluated in humans at that time. All animals were treated for 6 days, and followed as above for 34 days after treatment.

There was no predictable pattern of diarrhea or constipation for any mouse or group of mice, although stool consistency varied from loose to firm and dry. All animals gained weight during the experiment without noticeable difference between treatment groups. A single mouse developed a diffuse red punctate rash over its face, body, and legs one day after receiving its first 4 mg dose of diclazuril which resolved within 6 days. No other clinical abnormalities were noted. The mean number of Cryptosporidium oocysts (per 5 high power (45x) microscopic fields) excreted by each of the three treatment groups increased over time despite treatment although the increase was least in the group receiving the highest dosage. Twelve of 19 animals spontaneously died, with persistent cryptosporidiosis, between 93 and 123 days after initial infection; there was no clustering of deaths during any time period in any treatment group. The remaining mice were sacrificed.

Diclazuril, therefore, either 4 mg or 8 mg per day, administered for a six-day period, did not have demonstrable therapeutic efficacy in the adult athymic nude mouse model of chronic cryptosporidiosis although numbers of oocysts shed did decrease in treated compared to untreated groups. It is now

clear from concurrently performed human studies that similar dosages for similar time periods in infected patients are unlikely to be efficacious. Additional evaluation is planned.

D. Development of Mouse Models of Cryptosporidiosis and Identification of Immunologic Mediators of Infection

Initial work led to development of two mouse models of chronic symptomatic cryptosporidiosis, one in adult congenitally athymic nude mice and the other in anti-CD8+ lymphocyte and/or anti-CD4+ lymphocyte monoclonal antibody-treated suckling BALB/c mice (75). For the former, approximately 10^7 C. parvum oocysts were fed on two successive days to each of 21 adult athymic mice in three temporally unrelated experiments. In two other temporally unrelated experiments, neonatal BALB/c mice were fed approximately 10^7 oocysts on days 3 and 5 of life. (This dosing regimen was chosen to eliminate the variability noted in the ability of some C. parvum isolates to cause infection and illness.) All mice received a subcutaneous dose of 12 mg sulfadimethoxin of days of gavage.

Clinical assessment and fecal examination were performed on each mouse. Fecal pellets were examined for Cryptosporidium oocysts before feeding and at least four times per week thereafter. Stool consistency was noted. Each animal was also assessed clinically on days of stool collection for lethargy, weight loss, skin turgor, skin coloration and abdominal distention. Microbiologic and pathologic examination were performed at the conclusion of the experiment. Serum was analyzed for antibodies to the following murine pathogens: minute virus of mice, pneumonia virus of mice, reovirus type 3, mouse hepatitis virus, K virus, Theiler's virus, Sendai virus, lymphocytic choriomeningitis virus, mouse adenovirus, ectromelia virus, polyomavirus, and Mycoplasma pulmonis (Armed/Biosafe, Rockville, MD). At necropsy, the contents of the stomach, duodenum, ileum, jejunum, cecum, colon, and gallbladder were aspirated and examined for Cryptosporidium oocysts. A complete gross examination of all organs and tissues was conducted, and any visible abnormalities were noted. All major organs and tissues were collected and placed in 10% buffered Formalin for fixation, routine histopathologic processing, microscopic examination.

In adult athymic mice, clinical symptoms including dehydration, weight loss, and intermittent diarrhea, and elevated oocyst shedding developed by the fourth week. This picture resembles that seen in many HIV infected patients: waxing and waning diarrhea and oocyst passage. Most mice developed a progressive infection with Cryptosporidium, fatal within four months; a few animals developed stable infections. No other murine pathogen was identified in any animal. Pathologic abnormalities and organisms localized in the intestine in stable infections but involved the hepatobiliary tree including bile ducts, gallbladder and pancreas in progressive infections; this resembles histopathology found in some HIV-infected patients. Lymphoid cells from histocompatible, previously infected and therefore thought to be Cryptosporidium-immune mice cured infected nude mice.

In neonatally infected BALB/c mice which normally clear Cryptosporidium infection spontaneously within three weeks, treatment with monoclonal antibodies directed against CD4+ lymphocytes alone (GK 1.5; rat IgG2b anti-mouse CD4) or with monoclonal antibodies directed against CD8+ lymphocytes (2.43; rat IgG2b anti-mouse CD8) led to prolonged infection. Clinical findings, oocyst shedding

and necropsy results were similar to those described for the athymic model as long as CD4+ T-cell ablation continued. The mice were cured when these monoclonal antibody treatments were stopped. Deletion of CD8+T cells alone, in contrast, failed to prolong neonatal infection.

To define more specifically the immune defects which might permit establishment of Cryptosporidium infection in adult mice, adult BALB/c mice which are normally resistant to infection, were treated, prior to attempting Cryptosporidium infection, with individual or combinations of monoclonal antibodies directed against the CD4, CD8 or CD3 determinants of T-lymphocytes or against the lymphokines, gamma-interferon or interleukin-2 (manuscript in preparation).

The combination of GK 1.5 and 2.43 monoclonal antibodies directed against CD4+ and CD8+ T-lymphocytes or only monoclonal antibodies directed against CD4+ T-lymphocytes led to scant but continuous shedding of low numbers of Cryptosporidium oocysts without any clinical manifestations. Prolonged and diffuse Cryptosporidium infection did not occur in adult animals as it had in the easily infectable neonatal mouse model when similarly treated or in the adult athymic mouse model. In contrast, all animals receiving 2C-11, a monoclonal antibody directed against any T cell with CD3 determinants, developed intense infection within three weeks of Cryptosporidium inoculation. In each of the three temporally unrelated times this experiment was performed, some mice receiving 2C-11 developed severe cryptosporidiosis with autopsy findings similar to the other chronically infected mice. Other animals spontaneously decreased and then stopped shedding oocysts after approximately 5 weeks of 2C-11 administration. This is consistent with mouse antibody production to 2C-11 and subsequent neutralization of the monoclonal antibodies' effects. A final group of animals only cleared Cryptosporidium infection when 2C-11 monoclonal antibody was discontinued. In efforts to block possible antibody production to 2C-11, either dexamethasone or GK 1.5 was used in conjunction with 2C-11. Animals so treated shed larger numbers of Cryptosporidium oocysts earlier than mice treated with 2C-11 alone and developed diffuse cryptosporidiosis with necropsy findings comparable to those of infected adult athymic mice.

These experiments show that while T-lymphocytes (cells with CD3 determinants) are important mediators of intense Cryptosporidium infection in the adult mouse, absence of the CD4+ T-lymphocyte (with or without simultaneous absence or the CD8 T-lymphocyte) does not lead to fulminant cryptosporidiosis as it does in the less immunologically mature neonatal mouse. Lack of the CD4+ T-lymphocyte does, however, permit a chronic level of Cryptosporidium oocyst shedding without significant clinical manifestations or extensive pathological findings in animals who would otherwise be totally refractory to Cryptosporidium infection. These experiments suggests that a spectrum of clinical cryptosporidiosis exists in the murine model which correlates with increasingly extensive immunologic deficits; it may be comparable to the spectrum of clincial illness noted in HIV-infected patients who may have persistent cryptosporidiosis with modest symptoms or who may manifest relentless and fulminant illness with Cryptosporidium found diffusely in many organs and contributing to mortality. These findings furthermore suggest the possibility that some T-cell population, absent in the athymic mouse, which is either CD4- or resistant to the effects of anti-CD4 monoclonal antibody is important in preventing the establishment of severe Cryptosporidium infection but is insufficient to eradicate an already established infection.

A final set of experiments evaluated the effect of the two lymphokines, gamma-interferon and interleukin-2, in permitting severe cryptosporidiosis to develop. All animals in 4 separate experiments treated with the monclonal antibody XMG-6 (against gamma-interferon) developed significant Cryptosporidium infection within two weeks of inoculation which, however, was self-limited, again perhaps related to mouse antibody produced to XMG-6. Addition of GK 1.5, the monoclonal antibody directed against CD4+ T-lymphocytes led to persistent infection; at necropsy of a single animal sacrificed, diffuse cryptosporidiosis was found. In all other animals, Cryptosporidium oocyst excretion cleared once GK 1.5 monoclonal antibody was discontinued. These experiments suggest that decreased gamma-interferon may be important in establishment of initial but not persistent infection.

Monoclonal antibody directed against interleukin-2, in contrast, did not have any effect on initiation of Cryptosporidium infection: no animal had any detectable Cryptosporidium in fecal specimens oocysts following the initial pass through of the inoculated organisms. This was identical to findings in groups of mice used as controls in each experiment who were injected with monoclonal antibodies lacking any murine determinants.

The animal models described here are important in several respects: (1) they have identified that different defects may be important in establishment of initial infection and in production of chronic infection and that different defects may lead to chronic but not fulminant cryptosporidiosis; (2) they provide models which may be useful in assessment of potential prophylactic and treatment modalities; (3) they suggest that gamma-interferon may be useful as an interventional agent in limiting severe clinical illness and should be tested in animal models. These animal models of Cryptosporidium infection will continue to be useful in understanding the pathogenesis of Cryptosporidium infection and in identification of other immune defects that might be amenable to immunotherapy.

E. Other ELISA Tests

Conventional ELISA tests to detect antigens of Entamoeba histolytica or Giardia lamblia in fecal specimens are now truly well-established and have been developed by at least six groups for E. histolytica and three for G. lamblia (67-70, 76-82) substantiating efficacy. The Giardia assay originally developed in our laboratory (67) shown to detect antigens which are shared in common by at least eight distinct Giardia isolates (83). In one application of this ELISA, ELISA analysis of stool specimens obtained after oral inoculation of human volunteers with Giardia lamblia showed that 94.5% of those with cysts in their stools were positive by ELISA and that during treatment, the ELISA was significantly better than fecal examination in detection of Giardia lamblia antigen (84).

Field testing of this Giardia lamblia ELISA test was completed in Lima, Peru (85): Thirty children were prospectively followed, 15 with at least one microscopy stool examination positive for Giardia lamblia and 15 without Giardia lamblia during the first year of life. From these 30 children followed for one year, there was a mean of 38+/-6 stools per child for a total of 1131 fecal specimens. Giardia lamblia was detected by both ELISA and microscopy in 91 stools, by ELISA alone in 44 and by microscopy alone in 17. Episodes (defined as the beginning and the end of a period of Giardia lamblia excretion) were

detected with similar frequency by ELISA or stool microscopy. Another group of 17 hospitalized children that did not have Giardia lamblia detected on stool examination had stools examined by ELISA and a duodenal aspirate examined using microscopy. Six patients had Giardia lamblia detected, two only by ELISA, one only by duodenal aspirate and three by both examinations. In this study, the ELISA was as sensitive as stool microscopy for the detection of Giardia lamblia in stool. It also correlated well with duodenal fluid examination.

In another effort, reagents for the Entamoeba histolytica ELISA previously developed in our laboratory (68) were obtained and retested for optimal concentrations by checkerboard titration since the commercially-available specific monoclonal antibody had been modified since last used. Sensitivity and specificity were re-established using the parasitologically-known stool specimens, frozen at -70°C. This ELISA assay was used in an on-going study on the etiology of diarrheal disease among travelers, foreign residents and Peace Corps Volunteers in Nepal (86). More than 300 stool specimens from patients at two clinic locations were examined by microscopy in the field and by ELISA at USUHS after specimens were shipped frozen at -70°C. About 5% of patients from each clinic had ELISA detectable E. hisotolytica antigen, compared to 4% by microscopy at one clinic and 26% at another clinic thought to have significant problems in misdiagnosis. The correlation between microscopy and ELISA was approximately 50% at the first clinic suggesting that the ELISA may be a reasonable alternative diagnostic technique which should be tested directly in the field.

Although at least two commerical companies have taken preliminary steps towards producing marketable ELISA kits for each of these parasites, their utility outside a research laboratory setting remains to be fully demonstrated.

F. Publications

Treatment of Cryptosporidiosis with Hyperimmune Bovine Colostrum

1. Ungar BLP, Ward DJ, Fayer R and Quinn C: Successful Use of Hyperimmune Bovine Colostrum to Treat Cryptospiridium Infection in an Acquired Immunodeficiency Patient. Gastroenterology 98:486-489, 1990.

   Significance: Hyperimmune bovine colostrum has therapeutic benefit in an HIV-infected patient.

2. Fayer R, Andrews C, Ungar BLP and Blagburn B: Efficacy of Hyperimmune Bovine Colostrum for Prophylaxis of Cryptosporidiosis in Neonatal Calves. J Parasit 75:393-397, 1989.

   Significance: Hyperimmune bovine colostrum has prophylactic benefit in a calf model.

3. Moon H, Woodmansee D, Harp JA, Abel S and Ungar BLP: Lacteal Immunity to Enteric Cryptosporidiosis in Mice: Immune Dams do not Protect Their Suckling Pups. Infect Immun 56:649-653, 1988.

   Significance: Non-hyperimmune colostrum is not protective in a mouse model.

4. Ungar BLP, Kao DA and Burris JA: Use of a Murine Model of Cryptosporidium Infection to Evaluate Therapeutic Interventions (in preparation).

   Significance: In vivo models are now available for efficacy testing of chemotherapeutic and biologic agents.

Epidemiologic Parameters of Cryptosporidiosis

5. Ungar BLP, Mulligan M and Nutman TB: Serologic evidence of Cryptosporidium Infection in U.S. Peace Corps Volunteers Before and During Peace Corps Service in Africa. Arch Intern Med 49:894-897, 1989.

   Significance: Immunologically healthy individuals based in endemic areas have a high rate of seroconversion and are likely at risk for infection.

6. Hayes EB, Matte TD, O'Brien TR, McKinley TW, Logsdon GS, Rose JB, Ungar BLP, Ward DM, Pinsky PF, Cummings ML, Wilson MA, Long EG, Hurwitz ES and Juranek DD: Contamination of a Conventionally Treated Filtered Public Water Supply by Cryptosporidium Associated with a Large Community Outbreak of Cryptosporidiosis. NEJM 320:1372-1376, 1989.

   Significance: Water-borne outbreak affecting approximately 13,000 individuals on a common water supply is described.

7. Ungar BLP, Gilman R, Lanata C and Perez-Schael I: Sero-epidemiology of Human Cryptosporidium Infection in Two Latin American Populations. J Infect Dis 157:551-556, 1988.

   Significance: Cryptosporidium has a higher prevalence in less developed areas of the world and persons in these areas are likely at risk for infection.

ELISA Testing for Cryptosporidium, Entamoeba histolytica, Giardia lamblia

8. Ungar BLP: Enzyme-linked Immunoassay for the Detection of Cryptosporidium Antigens in Fecal Specimens. J Clin Micro (in press).

   Significance: A first generation ELISA test that has now been developed which will improve rapidity and accuracy of diagnosis.

9. Ravdin JI, Simjee AE, Petri WA, Murphy CF, Ungar BLP and Jackson TFHG: Comparison of Clinical Status, Zymodeme Analysis, and Serum Immunoblot Recognition of Entamoeba histolytica Antigens. J Infect Dis 162:768-772, 1990.

   Significance: More sensitive reagents may provide a refined and more useful ELISA test to detect Entamoeba histolytica antigens in fecal specimens.

10. Taylor DN, Houston R, Shlim DR, Echeverria P, Bhaibulaya M and Ungar BLP: The Etiology of Diarrheal Disease among Travelers, Foreign Residents, and Peace Corps Volunteers in Nepal. J Amer Med Assn 260:1245-1248, 1988.

Significance: ELISA testing for Entamoeba histolytica antigen in fecal diagnostic techniques in specimens of diverse geographic origins.

11. Vidal MFC, Gilman RH, Ungar BLP, Verastegui MR, Benel AC, Marquis G, Penny M, Lanata C, and Miranda E: Detection of Giardia lamblia Antigen in a Longitudinal Study of Children Living in a Peruvian Peri-Urban Shanty Town (Pueblo joven) (submitted).

Significance: In a field setting, ELISA testing offers a cheap, quick and reliable diagnostic tool which is as sensitive as microcopy and which is a practical alternative in epidemiologic studies.

Mouse Models of Chronic Cryptosporidium Infection

12. Ungar BLP, Burris JA, Quinn CA, and Finkelman FD: New Mouse Models for Chronic Cryptosporidium Infection in the Immunodeficient Host. Infect Immun 58:961-969, 1990.

Significance: First development of a mouse model of symptomatic cryptosporidiosis which mimics illness seen in HIV-infected patients; this can be used to test interventional agents and to define specific immune defects leading to chronic illness.

13. Ungar BLP, Finkelman FD, Kao DA, and Burris JA: Lymphocyte and Lymphokine Mediators of Cryptosporidium Infection in an Adult Murine Model (in preparation).

Significance: Regulation of initial Cryptosporidium infection and prolonged infection is through different mediators which will be important in development of effective therapy.

Other

14. Schlim DR, Cohen MT, Eaton M, Raja R, Long EG and Ungar BLP: An Algae-like Intestinal Organism Associated with an Outbreak of Prolonged Diarrhea Among Foreigners in Nepal (submitted).

Significance: A new putative agent of chronic diarrhea which may be diagnostically confused with Cryptosporidium is described.

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